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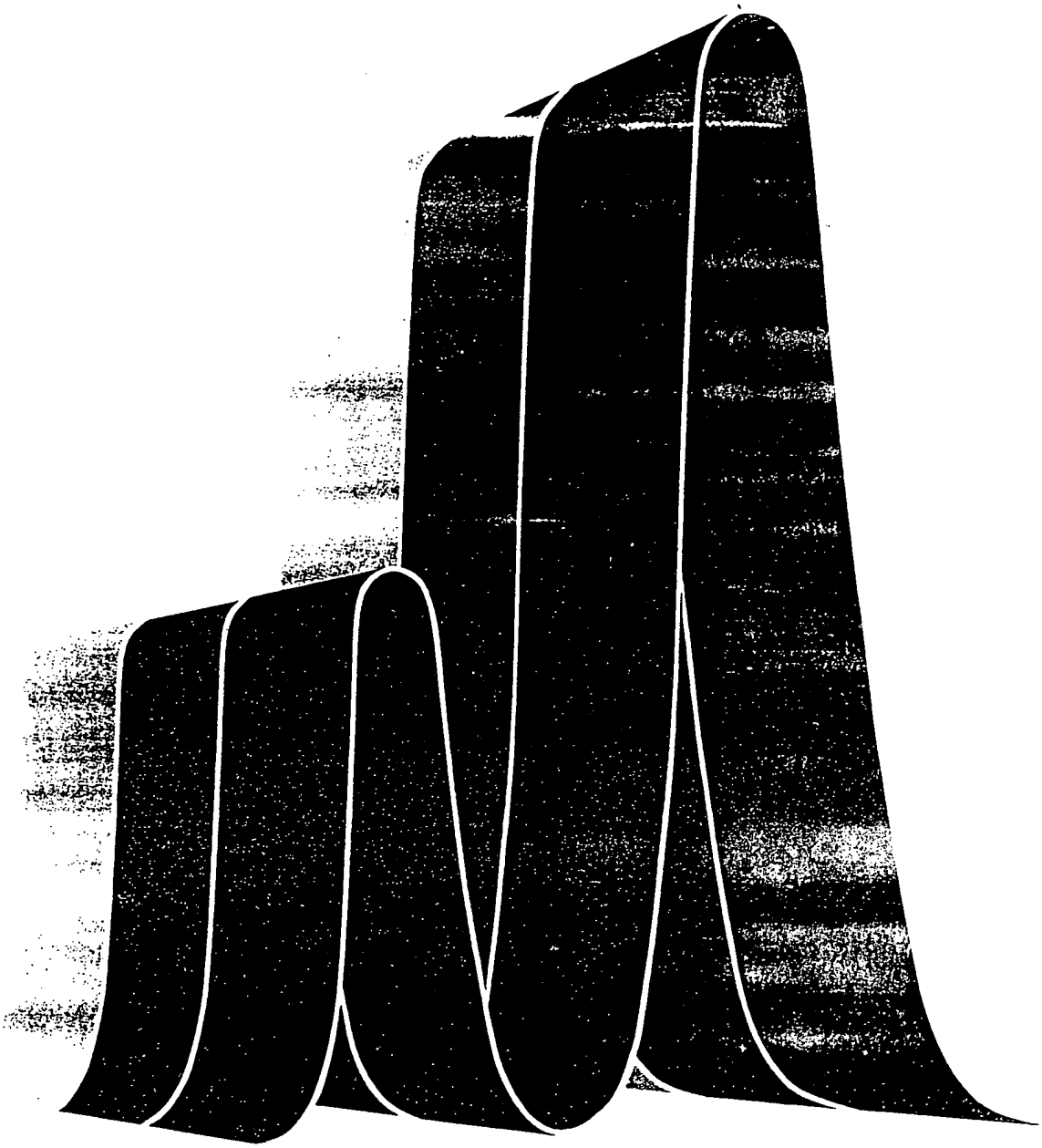
Gel filtration
theory and practice



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Principle

As a solute passes down a chromatographic bed its movement depends upon the bulk flow of the mobile phase and upon the Brownian motion of the solute molecules which causes their diffusion both into and out of the stationary phase. The separation in gel filtration depends on the different abilities of the various sample molecules to enter pores which contain the stationary phase. Very large molecules which never enter the stationary phase, move through the chromatographic bed fastest. Smaller molecules, which can enter the gel pores, move more slowly through the column, since they spend a proportion of their time in the stationary phase. Molecules are, therefore, eluted in order of decreasing molecular size (see pages 30—35 for a more detailed discussion).

the whole range making Sephacryl an excellent general purpose gel filtration medium. An example showing the separation of serum proteins is given in Figure 6.
Table 2 gives the fractionation ranges for the different S-types. Figure 7 shows the selectivity curves for globular proteins.

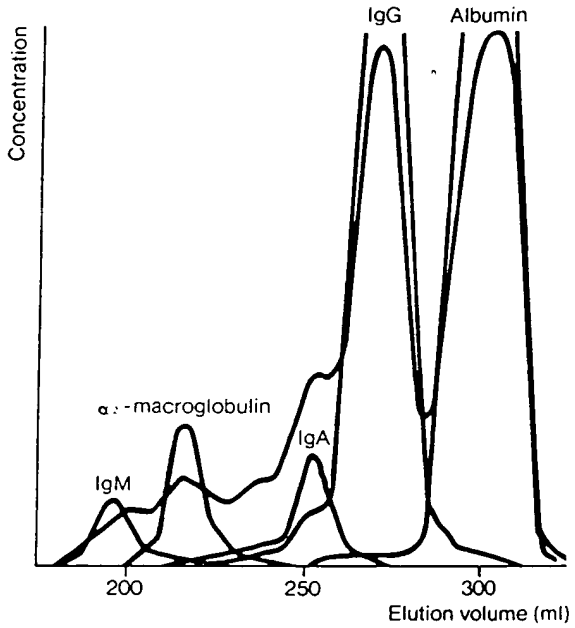


Fig. 6. Gel filtration of serum proteins on Sephacryl S-300 Superfine. Pharmacia Column K 26/100; bed height: 94 cm; eluent: Tris-HCl buffer solution (I = 0.1, pH 8.0) containing NaCl (0.5 M); flow rate: 2.4 ml . cm^{-2} . h^{-1}. (Work from Pharmacia Fine Chemicals).

Adsorption

The high matrix-density of Sephacryl gives it somewhat more pronounced adsorption properties than Sephadex of similar porosity. Adsorption is pH dependent and a number of solutes including Blue Dextran 2000 are adsorbed at pH 4. At pH's around neutrality yields approaching 100 % may be expected. Aromatic solutes may be retarded somewhat. For the best results eluents with an ionic strength of at least 0.05 should be used (10).

Availability

Sephacryl S-200 Superfine and S-300 Superfine are supplied pre-swollen, ready to use in packs of 750 ml as a suspension in distilled water containing 0.01 % Merthiolate®.

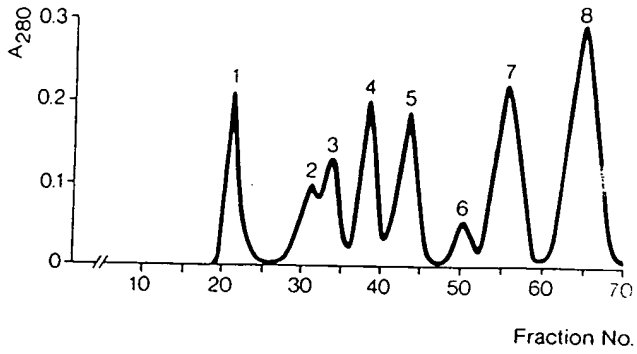


Fig. 15. Gel filtration of proteins on Sepharose CL-6B. Peaks: 1. Blue Dextran 2000; 2. bovine serum albumin; 3. rabbit IgG; 4. α-chymotrypsinogen; 5. cytochrome c; 6. insulin; 7. B chain of insulin; 8. DNP-Ala. Column: 1.5 × 90 cm; eluent: 6 M guanidine-HCl, 0.1 M sodium phosphate, pH 8.0; flow rate, 6.8 $ml.cm^{-2}.h^{-1}$; fraction size: 2 ml. (Ansari, A.A., Mage, R.G., J. Chromatogr. 140 (1977) 98—102. Reproduced by kind permission of the Authors and the Publisher).

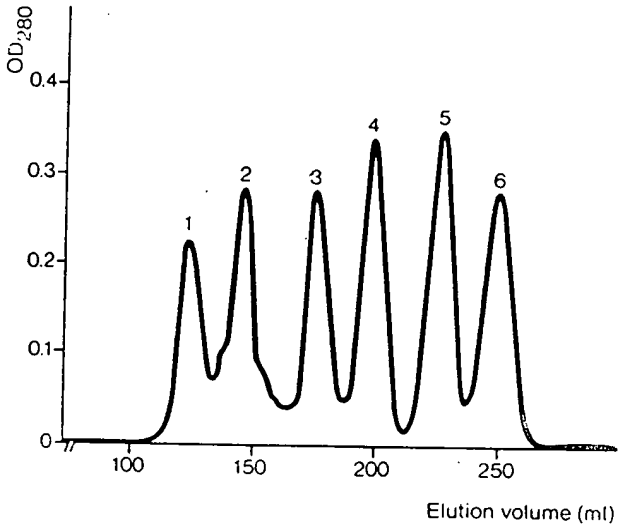


Fig. 16. Calibration kits for gel filtration. An example showing their use with Sephadex G-200 Superfine. Peaks: 1. catalase; 2. aldolase; 3. bovine serum albumin; 4. ovalbumin; 5. chymotrypsinogen A; 6. ribonuclease A. Pharmacia column: K 26/70; eluent: 0.05 M potassium phosphate, pH 6.8, containing 0.1 M NaCl and 0.02 % NaN_3; flow rate: 1 $ml.cm^{-2}.h^{-1}$. (Work from Pharmacia Fine Chemicals).

The only requirements are a column packed with the appropriate gel and a series of protein standards to calibrate it. Gel Filtration Calibration Kits HMW and LMW from Pharmacia Fine Chemicals provide a series of well-characterized globular protein standards carefully chosen to give reliable calibration points in the molecular weight range 13 700 to 669 000 (Table 7). An elution profile obtained with Sephadex G-200 Superfine is shown in Figure 16.

Table 7. Contents of the gel filtration calibration kits.

Low Molecular Weight Gel Filtration Calibration Kit

Protein	Molecular Weight	Stokes' Radius (Å)	Source
ribonuclease A	13 700	16.4	bovine pancreas
chymotrypsinogen A	25 000	20.9	bovine pancreas
ovalbumin	43 000	30.5	hen egg
albumin	67 000	35.5	bovine serum
Blue Dextran 2000			

High Molecular Weight Gel Filtration Calibration Kit

Protein	Molecular Weight	Stokes' Radius (Å)	Source
Aldolase*	158 000	48.1	rabbit muscle
catalase	210 000		bovine liver
ferritin*	440 000	61.0	horse spleen
thyroglobulin	669 000	85.0	bovine thyroid
Blue Dextran 2000			

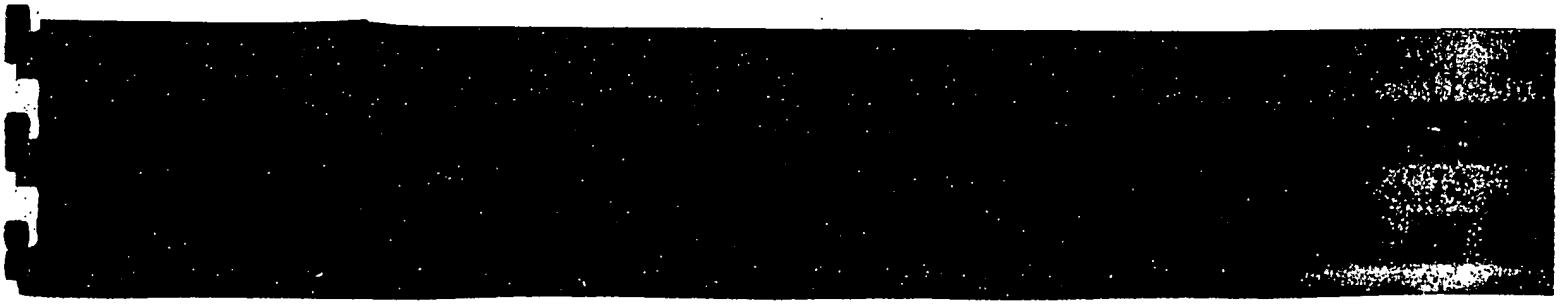
Each Kit contains 50 mg of each protein and 50 mg of Blue Dextran 2000.

*These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid in their solubilization. The percent of protein in each vial is indicated on the label. This quantity should be taken into consideration when making solutions of these proteins to apply to the column.

For the determination of molecular weights of small samples, thin-layer gel filtration may be used. The calibration curves in this case are very similar to those obtained in column experiments. Thin-layer gel filtration is described in a separate booklet available from your local supplier of Sephadex and other gel filtration media.

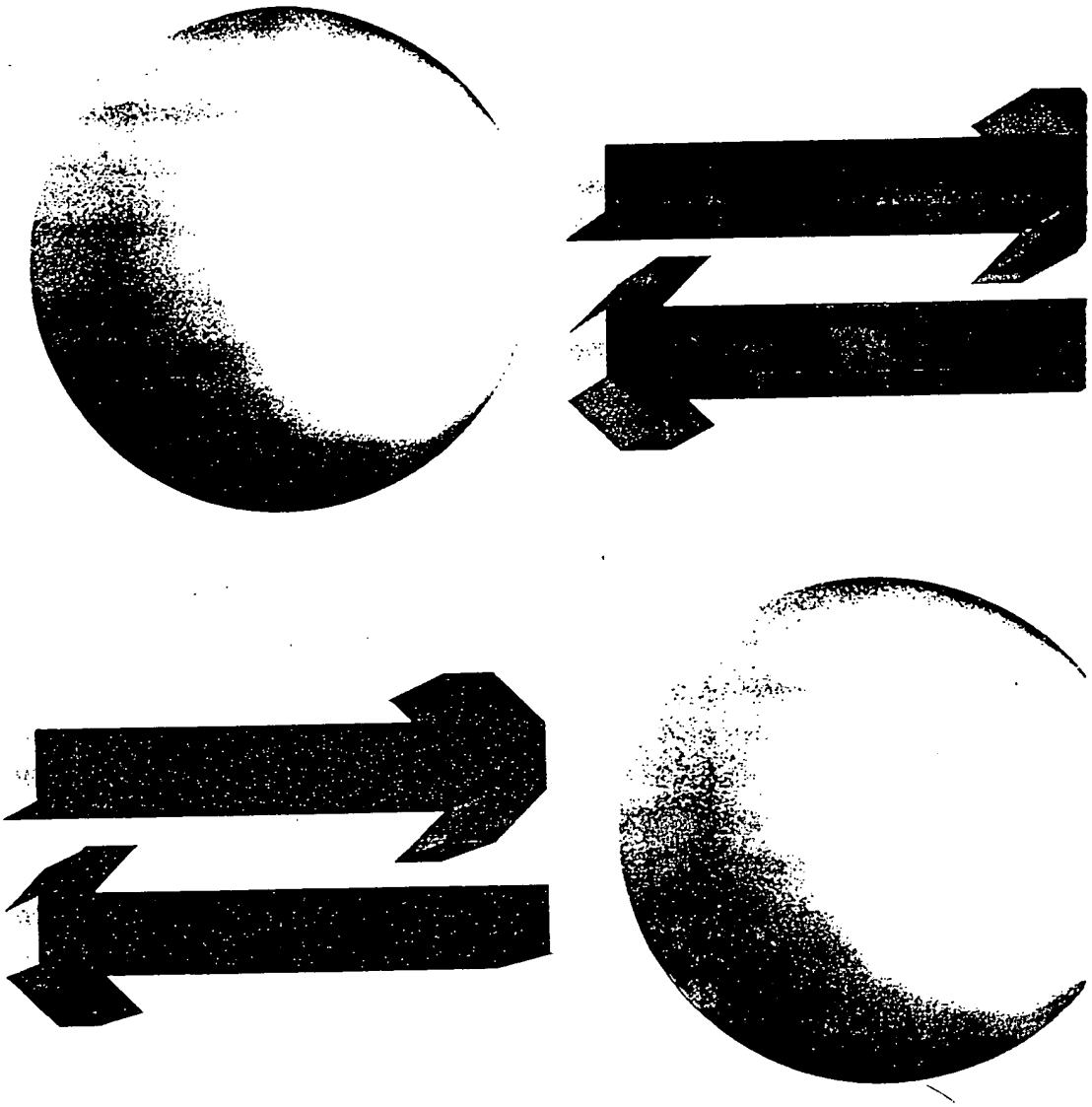
Determination of molecular weight distribution of polymers

The molecular weight distribution is very important for characterization of natural and synthetic polymers. Distribution analysis by classical methods is difficult and tedious as it involves fractionation of the macromolecules by precipitation and determination of molecular weight and amount of substance in every fraction.



Ion Exchange Chromatography

principles and methods



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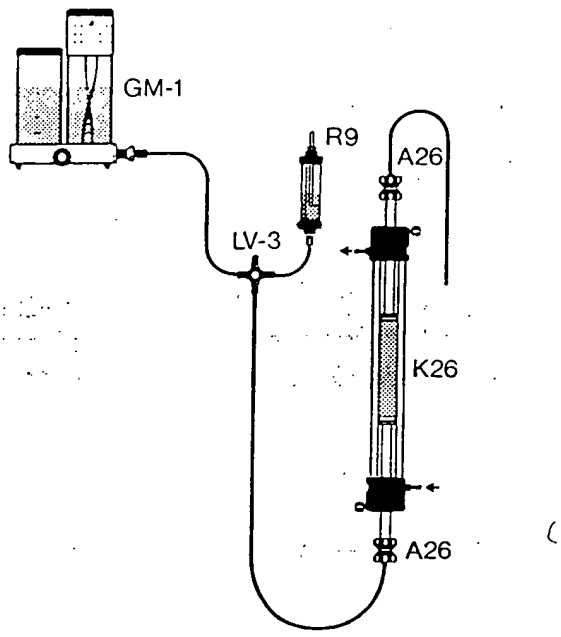

Fig. 20. Sample application using a reservoir. This is also an example of upward elution.

Sample reservoir (fig. 20). In a similar way, a sample reservoir (e.g. R9, R15/16) can be connected via a 3-way valve to apply larger samples.

Sample applicators SA-5, SA-50. These are reservoirs which allow the sample to be introduced as a layer below the eluent using a syringe and needle without disturbing the chromatographic bed. They can be used in a sample loop system (fig. 21) where their large capacity (up to 6 ml for the SA-5 and 45 ml for the SA-50) and lack of tailing, due to minimal wall effects, offer distinct advantages.
The column should be washed with approximately one bed volume of starting buffer before eluting substances of interest.

8.5 Elution

If conditions are chosen such that unwanted substances in the sample are adsorbed from the substance of interest, then no change in elution conditions are required since the substance of interest passes straight through the column. However, when conditions have been chosen so that the substances of interest are bound to the gel, elution can be achieved by varying either buffer pH, ionic strength, or possibly both. Occasionally conditions can be chosen such that sample components are separated by elution with starting buffer. This is termed isocratic elution, and the column is said to be developed by starting condition procedure. Isocratic elution is useful since

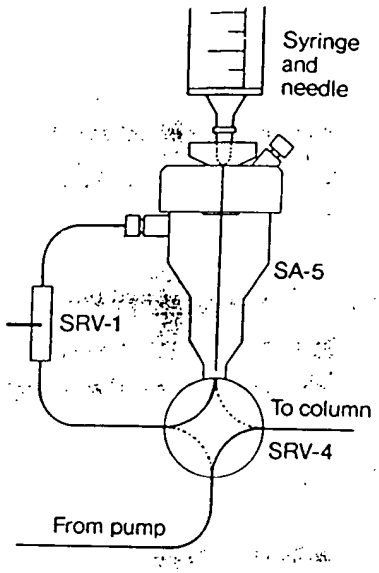

Fig. 21. Sample application using an SA-5 in a sample loop system.

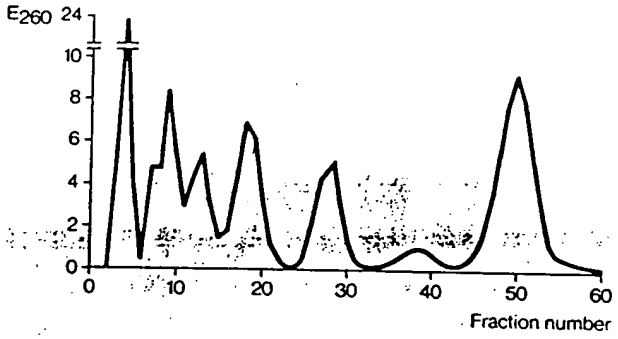

Fig. 22. Elution pattern of an extract of the eyes of the catfish on CM-Sephadex C-25. Sample: 3 ml. Column: 1.05×40 cm. Eluent: 0.5 M ammonium formate, pH 6.5. Flow rate: 24 ml.h^{-1} (27.7 cm.h^{-1}). Fraction size: 3.8 ml. (Ho, S., Nicol, J.A.C. Biochem. J. 153 (1976) 567—570. Reproduced by kind permission of authors and publisher).

no gradient apparatus is required for the run, and if all the retarded substances also elute, regeneration is not required. Figure 22 shows the separation of *S*-adenosyl-3-thiopropylamine and other basic compounds extracted from the reflecting material of the eye of the sea catfish on CM-Sephadex C-25 using isocratic conditions (17). The time of separation can be rather long using starting conditions and substances may be irreversibly bound, so it is normally desirable to alter some of the conditions to achieve elution.

Change of pH

As shown in Figure 17, the net charge on a molecule is dependent on pH. Thus altering the pH towards the isoelectric point of a substance causes it to lose its net charge, desorb, and elute from the ion exchanger. Figure 23 shows that arthropod haemocyanin treated with 1 M urea can be separated into six fractions on DEAE-Sepharose CL-6B using a decreasing pH gradient (18).

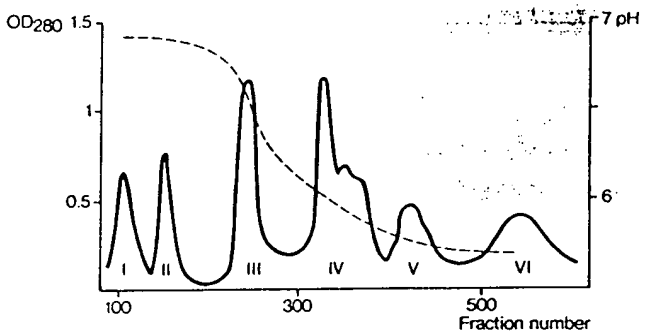

Fig. 23. Elution pattern of whole stripped haemocyanin on DEAE-Sepharose CL-6B. Sample applied in 0.1 M sodium phosphate buffer pH 6.8 and eluted by a descending pH gradient. (Lamy, J., Lamy., J., Weill, J. Arch. Biochem. Biophys. 193 (1979) 140—149. Reproduced by kind permission of the authors and publisher).

Change of ionic strength

At low ionic strengths, competition for charged groups on the ion exhanger is at a minimum and substances are bound strongly. Increasing the ionic strength increases competition and reduces the interaction between the ion exchanger and the sample substances, resulting in their elution. Figure 24 (overleaf) shows the elution pattern of phytohaemagglutinin isolectins on SP-Sephadex using a linear concentration gradient of 0—0.188M NaCl (19). Note that it is important to wash the column with starting buffer before starting the gradient.

Choice of gradient type

The components in the sample usually have different affinities for the ion exchanger and so variations in the pH and ionic strength of the eluent can cause their elution at different times and thus their separation from each other. One can choose to use either continuous or stepwise gradients.

Continuous pH gradients are difficult to produce at constant ionic strength, since simultaneous changes in ionic strength, although small, also occur. Linear pH gradients cannot be obtained by mixing buffers of different pH in linear volume ratios since the buffering capacities of the systems produced are pH dependent. In addition, the buffer has to titrate the buffering action of the ion exchanger.

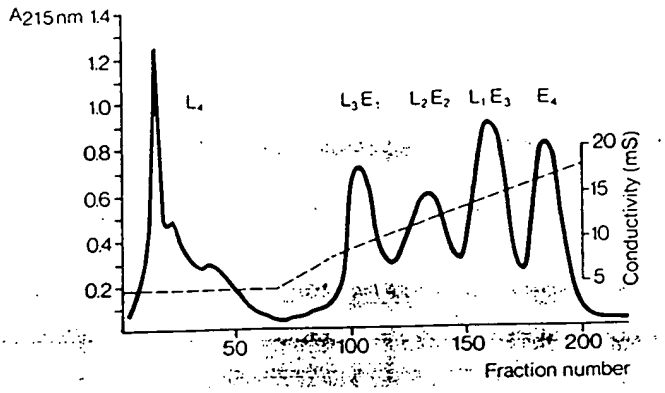

Fig. 24. Elution pattern of mixtures of phytohaemagglutinin isolectins on SP-Sephadex. Column: 1.5 × 45 cm. Eluent: 0.05 M potassium phosphate, pH 6.0, with a linear salt gradient to 0.188 M NaCl. Fraction size: 5 ml. L = lymphocyte reactive subunit, E = erythrocyte reactive subunit (Felsted, R.L., Egorin, M.J., Leavitt, R.D. et al. J. Biol. Chem. 252 (1977) 2967—2971. Reproduced by kind permission of the authors and publisher).

Stepwise pH gradients are easier to produce and are more reproducible than linear pH gradients. Again the buffer must titrate the ion exchanger and there will be a short period of re-equilibration before the new pH is reached. pH gradients can be used in combination with ionic strength gradients.

Continuous ionic strength gradients are easy to prepare and very reproducible. Two buffers of differing ionic strength are mixed together and if the volume ratio is changed linearly, the ionic strength changes linearly.

Stepwise ionic strength gradients are produced by the sequential use of the same buffer at different ionic strengths.

The direction of gradients is summarized in Table 9.

Table 9. Choosing the direction of the gradient for elution.

Ion exchanger	Direction of pH gradient	Direction of ionic strength gradient
Anion exchanger	decreasing	increasing
Cation exchanger	increasing	increasing

Stepwise elution is technically simpler but has its disadvantages. Substances eluted by a sharp change in pH or ionic strength tend to elute close together and so peaks have sharp fronts and pronounced tailing since they contain more than one component. Tailing may lead to the appearance of false peaks if a buffer change is introduced too early. Linear gradients help substances to be eluted in symmetrical peaks and give better resolution. The differences between continuous and stepwise gradient elution are shown in Figure 25.

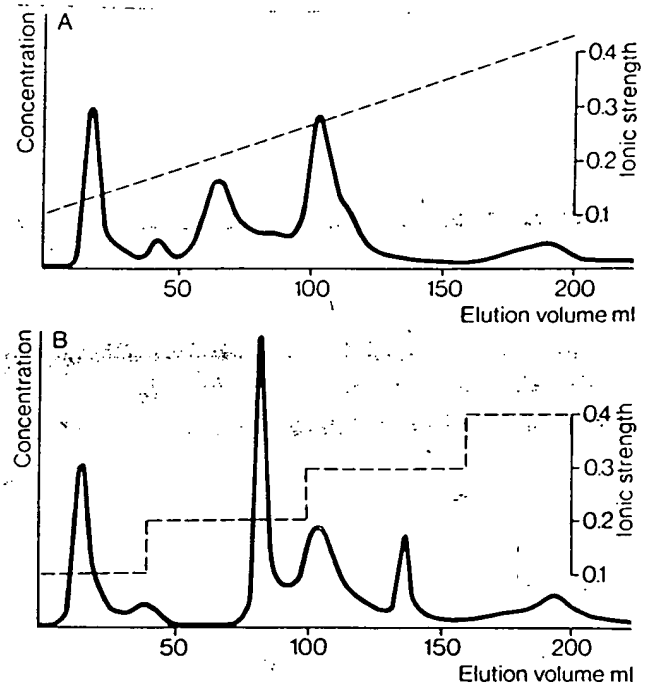

Fig. 25. Continuous and stepwise gradient elution of bovine serum on QAE-Sephadex A-50. Bed dimensions: 1.5 × 26 cm (column K 15/30). Sample: 4 ml 3% (w/v) lyophilized bovine serum. Eluent: 0.1 M Tris-HCl buffer, pH 6.5. Curve A was obtained using a continuous NaCl gradient to 0.5 M NaCl. Curve B was obtained using a stepwise NaCl gradient. Flow rate: 0.2 ml/min. The first peak in both diagrams represents IgG. The fourth peak in curve A represents serum albumin. Stepwise elution caused the albumin to be eluted in two peaks (4 and 5). Other peaks were not identified. (From Pharmacia Fine Chemicals, Uppsala, Sweden).

The total volume of eluent in a gradient should be about five times the bed volume of the ion exchanger. Longer gradients may lead to excessive band spreading and dilution, whilst shorter gradients may not give adequate separation. The steepness of gradients in terms of increase in ionic strength as opposed to volume must also be borne in mind (see page 53).

Gradient elution generally leads to good resolution because during elution zone sharpening occurs. In the absence of a gradient, one of the factors which would contribute to poor resolution would be band spreading as a result of diffusion. In gradient elution, the leading edge of a peak is retarded if it advances ahead of the concentration or pH required to elute it. Thus the trailing edge of the peak has a relatively higher speed of migration, resulting in zone sharpening.